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with a Potential Role in Tumorigenesis and Cancer Metastasis

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FOREWORD

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(5) Introduction:

One of the deadliest and least understood aspects of cancer is metastasis. Before a tumor can metastasize, individual cells must acquire mutations which down-regulate adhesion to neighboring cells. A number of studies have shown that down-regulating components of the adherens junctions, one of the primary cell-cell adhesion systems, causes increased invasiveness and metastatic potential of tumors (reviewed in (Behrens, 1999).

In adherens junctions, the extracellular domains of cadherins form homotypic interactions with neighboring cells (reviewed in (Tepass et al., 2000). The cytoplasmic tail of cadherin interacts with a class of proteins termed catenins, which associate with the actin cytoskeleton. Regulation of adhesion is an important process for cellular rearrangements such as during development and axon neurogenesis. p120 catenin is a candidate molecule that may function as a modulator of cell adhesion through its interaction with the membrane proximal region of the cadherin cytoplasmic tail (reviewed in (Reynolds and Daniel, 1997). In vertebrates p120 catenin is phosphorylated by tyrosine kinases in response to a variety of growth factors. Correlated with p120 catenin phosphorylation is a breakdown of adherens junctions, rearrangement of the actin cytoskeleton, and a loss of cell-cell contacts. p120ctn becomes highly tyrosine phosphorylated in metastatic, non-adherent cells. We are studying p120 catenin in *Drosophila* where we can take advantage of the many well-characterized tools in this genetic model system. *Drosophila* is an excellent model because flies have adherens junctions which are highly homologous to vertebrate adherens junctions at the molecular level. In addition, we have identified only a single p120ctn homolog in flies, and, with the completion of the genomic sequence, are reasonably certain that there are no others. The objective of this research project is to characterize the role of p120ctn by generating flies mutant for the p120 gene and characterizing them phenotypically and biochemically.

Specific Aim I. Intracellular localization and interaction of *Drosophila* p120ctn with *Drosophila* E-cadherin and other catenins

Specific Aim II. In vivo structure/function analysis of Dp120ctn

Specific Aim III. Transfer of knowledge gained in *Drosophila* to the study of breast cancer.

(6) Body:

Work on this project has been a collaboration between Rob Cavallo, the original awardee, who received his Ph.D. in December 1999, Gordon Polevoy, the PI from May 2000-April 2001, and Steve Myster, a postdoctoral fellow in the Peifer lab.

Mr. Polevoy took over as PI in May 2000. As I have discussed with Dr. Kandasamy and Ms. Blossom Widder, Contract Specialist, in the subsequent months, a series of events occurred which altered our plans. In June 2000 Mr. Polevoy became seriously ill and had to be hospitalized. When he was released from the hospital, the doctors recommended that he take time off from school and recover at home in Ohio, which he did, taking a leave from the University. At that point, it was not clear how long his leave of absence would last, and so we did not make any changes in our plans. However, while recovering, Mr. Polevoy decided not to continue in the Ph.D. Program. Instead, he returned for the spring semester, wrote a Master's thesis, and graduated with a M.S. in April 2001. This had several consequences for the grant. First, the project was delayed. Progress has not halted, as Mr. Polevoy was working with a post-doctoral fellow, but it has obviously slowed in Mr. Polevoy's absence. Second, as Mr. Polevoy has graduated, I (Mark Peifer, mentor of Mr. Polevoy) am writing this report. Third, we have an unspent balance of ~\$14,000. I formally requested a one year no-cost extension and identified a new student as a replacement PI. She is Ms. Lisa Swanhart, entering our program in August, when she will begin work on this project. This request is currently being considered by your agency for approval.

Specific Aim 1 Intracellular localization and interaction of *Drosophila* p120ctn with *Drosophila* E-cadherin and other catenins

p120 specific antibody

The p120 C-terminus was used to generate antibodies in both rabbits and rats. On western blots the antibodies are specific for a doublet that migrates between 90 and 100 kD. These

antibodies have been tested on embryos using immunofluorescence confocal microscopy. Dp120 is ubiquitously expressed and localizes to cellular junctions (Fig. 1A). There is also a fair amount of cytoplasmic staining. Interestingly, in early stage embryos a pair of bright staining structures have also been identified—these appear to be centrosomes (see below). The pattern of p120 intracellular accumulation closely resembles that we observe with a myc-tagged p120, using an antibody against the epitope tag (Fig. 1B; see below). Later in development, p120 becomes concentrated in the axons of the central nervous system (Fig. 1C), as does Armadillo (Loureiro and Peifer, 1998). We are continuing to characterize the pattern of p120 accumulation.

Myc-epitope-tagged p120

We have generated two different p120 transgenes that encode Dp120 with an added N-terminal 6xmyc-epitope. These have been used to generate transgenic *Drosophila*—we have 7 independent lines under control of the ubiquitin promotor, resulting in ubiquitous expression, and one line under the control of the GAL4-UAS system (Brand and Perrimon, 1993). We are currently generating additional lines of this transgene. The myc-tagged p120 is recognized both by our polyclonal anti-p120 antibodies (confirming their specificity) and by a monoclonal antibody against the myc-epitope. While different lines are expressed to different levels, in general the ubiquitin promotor drives expression at levels similar to those of endogenous p120.

We have also used these lines to examine the subcellular localization of p120, using the monoclonal antibody to the myc-epitope. p120 localizes to cell-cell junctions (Fig. 1B,D), co-localizing with both DE-cadherin (Fig. 1D) and Armadillo (data not shown). The staining is quite similar to that seen with our anti-p120 polyclonal antibodies (Fig. 1B,C), although the level of cytoplasmic staining is lower than that seen with the polyclonal anti-p120 antibodies. We can also detect accumulation of p120 in the central nervous system using the anti-myc antibody (Fig. 1C). Finally, we have used the myc-epitope to immunoprecipitate (IP) p120. It works well for this, and preliminary results suggest that it co-IPs DE-cadherin, Armadillo, and alpha-catenin, consistent with p120 being part of the adherens junction complex.

GFP-tagged p120

We have also generated a p120 transgene as a C-terminal GFP-fusion. The initial transgene lacks the C-terminal 54 amino acids of p120 (for ease of cloning), but we are currently generating a transgene with a fusion of GFP to full-length p120. These have been used to generate transgenic *Drosophila*—we have eight lines under control of the ubiquitin promotor, and five line under the control of the UAS-GAL4 system. The GFP-tagged p120 is recognized by anti-GFP antibodies, but is not recognized by our polyclonal anti-p120 antibodies, suggesting that either their epitope is in the C-terminal 54 amino acids that were truncated in cloning or that the C-terminal GFP fusion blocks the epitope by steric hindrance.

We used this GFP-tagged p120 to examine the subcellular localization of p120 both in living and in fixed embryos (Fig. 2). It gives a very strong signal in living embryos, and strongly labels cell-cell junctions (Fig. 2A-C). It also localizes to the developing tracheae (Fig. 2D), the axons of the central nervous system (Fig. 2E), and to the sense organs of the peripheral nervous system (Fig. 2F). We are currently using the p120 GFP fusion to examine the dynamic behavior of p120 during the complex morphogenetic movements of embryogenesis, including germband extension and dorsal closure, by taking time-lapse movies of these processes using our spinning disc confocal microscope. The p120-GFP also accumulates in paired structures within each cell—double labeling with antibodies to the centrosomal protein centrosomin reveal that these are centrosomes (Fig. 2G).

Specific Aim 2 In vivo structure/function analysis of Dp120ctn

Mapping p120 to a particular region of the 2nd chromosome using deficiencies

The *Drosophila* p120 gene was previously mapped to the 41C region of the right arm of the second chromosome near the centromere by hybridizing a *Dp120* cDNA probe to an ordered P1 genomic library. To more finely map the *Dp120* gene, deficiency strains and balanced stocks that contain mutations that also map to the 41C region were obtained from the Bloomington stock center. Complementation tests were performed to order the deficiencies in relation to each other and in reference to the genetic markers. In preparation for the *Dp120* genetic screen (see below) we performed three experiments designed to determine which of the deficiencies removed the *Dp120* gene. Initially, *in situ* hybridization of polytene chromosomes heterozygous for the deficiency were probed with the *p120* cDNA. To confirm these results

embryos were collected from the outcrossed deficiency strains and RNA *in situ* analysis was performed using an RNA probe generated from the *p120* cDNA. One-fourth of the embryos are homozygous for the deficiency chromosome and in strains that remove the *p120* gene we saw no *Dp120* transcripts in late stage embryos. Taken together (Fig. 3), these studies revealed that deficiency strains *Df(2R)M41A8*, *Df(2R)M41A4*, *Df(2R)Nap13*, and *Df(2R)Nap14* remove the *p120* gene and *Df(2R)Nap1*, *Df(2R)Nap2*, *Df(2R)Nap5*, and *Df(2R)Nap9* do not remove *p120*.

One problem that has slowed progress is that *p120* is at the junction of euchromatin and heterochromatin, in a region rich in repetitive DNA and where genes are embedded in these repetitive sequences. To refine the chromosomal position and to connect the physical and genetic maps (Fig. 3), embryonic progeny of parents heterozygous for various Deficiencies were collected. Once again one-fourth of the embryos are homozygous for the deficiency—we used GFP-marked Balancer chromosomes to select homozygous mutant embryos from their siblings. DNA was isolated from multiple single embryos and analyzed by PCR using two different sets of primers from *p120* as well as primer sets for several other genes in the region. This allowed us to determine whether *p120* or the other genes were removed by particular Deficiencies (Fig. 3). These studies revealed that deficiency strains *Df(2R)M41A8*, *Df(2R)M41A4*, *Df(2R)345*, *Df(2R)Nipped D*, and *Df(2R)Nipped E* remove the *p120* gene while *Df(2R)Nap1*, *Df(2R)Nap5*, and *Df(2R)41Ae* do not remove *p120*. They also showed that the *p120* gene is present in *l(2)41Af* and *l(2)NC21*. Finally, they placed the right endpoint of *Df(2R)345* between the genes *CG17528* and *TpnC-41C*, placed the right endpoints of *Df(2R)Nipped D* and *Df(2R)Nipped E* between the genes *CG2905* and *Ogt*, and the right endpoint of *Df(2R)M41A8* between the genes *Ogt* and *apterous*. This allows us to correlate the physical and genetic maps, and also suggested that candidate genes for two of the other complementation groups derived from our screen—*Nipped A* may correspond to *CG2905* and *l(2)Af* may correspond to *CG17258*.

Genetic screen to generate and identify mutations in the *p120* gene

As we previously reported, we carried out an F2 recessive lethal screen to identify EMS induced mutations that are lethal over deficiency *Df(2R)M41A8* (aka *Df740*). We screened 6,194 chromosomes and recovered 200 new mutants. We have taken advantage of two additional deficiency strains that overlap the deficiency used in the screen (Fig. 2). *Df(2R)M41A10* (aka *Df741*) removes *p120* and extends proximal toward the centromere. *Df(2R)Nap1* does not remove *Dp120* and extends distally. Each of the new mutants was tested for complementation against these strains and resulted in four subgroups (Fig. 3). Mutant strains in the first class complement *Df(2R)741* and fail to complement *Df(2R)Nap1*. These cannot be mutations in the *p120* gene because *Df(2R)Nap1* does not remove the *p120* gene. The second group comprises strains that fail to complement both deficiency strains. Mutants in the third class complement *Df(2R)Nap1* and fail to complement *Df(2R)741*. New *p120* mutations will be in this class. A fourth class was complemented by both deficiency strains. Mutations which met these criteria may represent mutations elsewhere on the second chromosome that result in lethality in combination with the chromosome carrying *Df(2R)740*, which was used in the screen.

Identification of the *Dp120* complementation group

We have further ordered the mutations in this region by using a set of very small deficiencies in the region. We have determined that *Df(2R)Nipped D*, *Df(2R)Nipped E*, and *Df(2R)345* remove the *p120* gene while *Df(2R)Ae*, and *Df(2R)Nipped C* do not remove *p120*. Thus the smallest Deficiency interval that contains *p120* is the overlap between *Df(2R)Nipped D* and *Df(2R)345* (Fig. 3). We have carried out complementation tests between these deletions and the NC mutations in the region. We have defined two complementation groups in this minimal region, which are *l(2)41Af*, of which we have at least three alleles, as well as the cloned gene *Nipped B* (Rollins et al., 1999), of which we generated 18 new alleles. There are also thirteen other mutations in the region, which are still being analyzed for complementation. One unusual feature of our screen is that a fairly large number of mutations derived from our screen appear to be deletions that remove more than one complementation group. Results obtained in an earlier genetic analysis of this region, which were initially interpreted to suggest complex interallelic complementation patterns, may also be explained by the generation of many deletions by EMS mutagenesis (Hilliker, 1976). EMS mutagenesis does not usually result in a high frequency of deletions—however, perhaps the highly repetitive nature of the DNA in the

region in question predisposes to the creation of deletions due to mispairing between adjacent repeats during the repair process.

We are using two approaches to identify whether any of our mutations represents mutations in the *p120* gene. In the first approach we are sequencing the coding region of the *p120* gene from a representative of each complementation group. The *p120* gene is comprised of four exons and the PCR products span the entire coding region of the *p120* gene except the first exon, that is predicted to encode the first seven amino acids of the p120 protein. The splicing donor and acceptor sequences of the second, third and fourth exons of the *p120* gene are present in the PCR products (we have not sequenced the 5' regulatory region). All of the PCR products were cloned and sequenced by our sequencing facility. Individual candidate mutations were reexamined by sequencing an independent clone to rule out PCR error. We have thus far sequenced the *p120* gene from the following alleles generated in our screen: *NC79*, *NC37*, *NC38*, *NC70* (all single allele groups), *NC1* (an allele of *l(2)IR3*), *NC9* (an allele of *l(2)IR23*), *NC19*, *l(2)Af*, *NC21*, *NC6* (an allele of *Nipped B*), and *NC3* (an allele of *Nipped A*). In none of these cases did we find a mutation altering the *p120* coding sequence or splice junctions. We are continuing to amplify, clone and sequence the remaining candidates.

The second approach utilizes the myc-tagged *p120* transgene driven by the ubiquitin promoter. The transgene maps to the third chromosome and is homozygous viable. Western blots show that the transgene is expressed at roughly comparable levels to the wild-type protein. This experiment has the caveat that the myc-tag might affect protein function, or that the ubiquitin promoter might not drive expression in all the correct tissues—ubiquitin promoter-driven expression has rescued a number of other genes, however. We placed this transgene into the background of a Deficiency that removes *p120* over a Balancer chromosome. We have crossed this strain to balanced stocks of many of our mutant strains, and have looked for rescue of the mutation to adult viability, as assessed by the presence of non-Balancer offspring. Strains tested include *NC19*, *NC21*, *NC29* (an *NC19* allele), *NC48* (a *NippedB* allele), *NC51* (an *NC19* allele), *V66*, *NC98* (an *NC19* allele), *V124*, *NC167*, *V189*, *V190*, *NC195*, *V202*, *V218*, *NC220*, and *l(2)Af*. Thus far none of the mutations tested was rescued by the transgene—we continue to test additional candidates.

Using these two approaches, we have now eliminated many of the candidates for mutations in *p120*, opening up the possibilities that: a) mutations in *p120* are not represented in our collection, and/or b) mutations in *p120* are not zygotically lethal. We are thus beginning to implement other approaches to abrogate p120 function. First, we have designed reagents to target *p120* for inactivation by double-stranded RNA interference (RNAi), an approach which has now been successfully used to inactivate a number of different fly genes (e.g., (Kennerdell and Carthew, 1998). We have designed templates to generate double-stranded RNAs against two different regions of *p120*. We have also made templates to RNAi *ftz*, a positive control used by many groups in setting up the RNAi system, due to its striking phenotype, and to *armadillo* and *DE-cadherin*, as positive controls for other junctional components. We hope to begin injecting these soon. We are also creating the stocks necessary to carry out a new screen for both zygotic lethals and female steriles over Df(2R)345.

Specific Aim III Transfer of knowledge gained in *Drosophila* to the study of breast cancer.

We have initiated a collaboration with the group of Keith Burrridge, to examine the function of fly p120 in cultured mammalian cells. They previously found that over-expression of mammalian p120 has a dramatic effect on cell morphology, and that this appears mediated in part by an interaction of p120 with the proto-oncogene Vav2, which acts as a GEF for Rho family GTPases (Noren et al., 2000) –similar results have been obtained by the Reynolds lab (Anastasiadis et al., 2000).

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Training accomplishments

Rob Cavallo, the original PI of this grant, defended his thesis in December 1999, and received his Ph.D. His thesis was composed in part of the work described above, and thus was supported in part by this grant. Rob presented the work to date at the Era of Hope Meeting in Atlanta in June 2000. A new graduate student, Gordon Polevoy, took over from Rob on the project, and this transfer was approved by the relevant officials of the USAMRMC. As noted above, Mr. Polevoy graduated with an M.S. degree in April 2001.

Appendix 1: Key research accomplishments.

- a) We have generated two different polyclonal antibodies which recognize p120 both on immunoblots and in situ in fixed embryos. P120 localizes to the cytoplasm and the cell-cell adherens junctions.
- b) We have generated two different myc-tagged p120 transgenes under the control of different promoters. Both are expressed and the ubiquitin promoter drives expression at levels similar to that of the endogenous promoter. We have used the epitope tag for in situ localization, confirming our work with the p120 antibody. We have also used it to immunoprecipitate p120 and in preliminary experiments have found that it co-IPs with other adherens junction proteins.
- c) We have generated two different GFP-tagged p120 transgenes under the control of different promoters. Both are expressed and allow us to visualize p120 in living embryos.
- d) We have mapped *p120* to a small region of the right arm of the second chromosome and correlated the genetic and physical maps in the region.
- e) We have isolated 200 lethal and visible mutations in the *Dp120* region.
- f) We have sorted candidates by complementation tests to deficiencies in the region.
- g) We are testing candidate mutations by sequencing and by attempting rescue with a transgene carrying wild-type *p120*

Appendix 2: Reportable outcomes in the entire 3 years.

Presentations discussing this work.

"Characterization of the *Drosophila* homolog of p120ctn, a modulator of adherens junctions." S.H. Myster, R. Cavallo, M. Peifer. 41st Annual *Drosophila* Research Conference, Pittsburgh PA, April 2000.

"The cloning and characterization of a *Drosophila* homolog of the adherens junction protein p120CTN." R. Cavallo, S.H. Myster, M. Peifer. Era of Hope, the DOD Breast Cancer Research Program Meeting, Atlanta GA June, 2000.

Degrees supported in part by this work

Ph. D. in Biology Awarded to Rob Cavallo, December 1999, Title: "New Partners for Armadillo in Signal Transduction and Cell Adhesion".

M.S. awarded to Mr. Gordon Polevoy, April 2001, entitled "Mechanisms of Armadillo's roles in signaling and adhesion." Virtually all the work in this thesis was supported by the IDEA Award.

Appendix 3-- Figures

Figure 1. p120 localization during embryogenesis. Embryos were fixed and stained with the indicated antibodies. A. Anti-p120 antibodies reveal that p120 localizes to both cell junctions and to the cytoplasm. A stage 9 embryo was double stained with antibodies to p120 (left panel, green in composite) and to the adherens junction protein Armadillo (middle panel, red in composite). The two proteins co-localize to cell-cell junctions. B, C. p120 antibody and antibody to the myc epitope reveal similar patterns of p120 accumulation. Embryo expressing myc-p120 under the control of the ubiquitin promotor double-stained with antibodies to p120 (left panel, green in composite) and to the myc-epitope (middle panel, red in composite). B. Stage 9 embryo showing co-localization to the adherens junctions. C. Stage 15 embryo showing co-localization to the axons of the central nervous system (arrow). D. Antibody to the myc-epitope specifically recognizes myc-p120, which co-localizes with the junctional protein E-cadherin. Stage 10 embryo expressing UAS-driven myc-p120 in alternating body segments, using the paired-GAL4 driver. The embryo was double-labeled with antibody to the myc-epitope (left panel, red in composite) and antibody to DE-cadherin (middle panel, green in composite).

Figure 2. p120-GFP localization during embryogenesis. A-F. Embryos expressing p120-GFP were imaged live, using a Perkin-Elmer Spinning disc confocal microscope. A. Cellularizing embryo showing that p120-GFP localizes to cell junctions and to two dots in the cytoplasm (arrow). B. Stage 14 embryo showing p120-GFP localization to cell-cell junctions of both the epidermis and amnioserosa, and its enrichment at the leading edge of the cells migrating during dorsal closure (arrow). C. Stage 15 embryo showing p120-GFP localization to cell-cell junctions of epidermal cells and its enrichment in sense organs of the peripheral nervous system (arrow). D. Stage 14 embryo showing p120-GFP enrichment in the developing tracheal system (arrow). E. Stage 15 embryo showing strong enrichment of p120-GFP in the axons of the developing central nervous system (arrow). F. Stage 15 embryo showing strong enrichment of p120-GFP in the sensory elements of the chordotonal organ (bracket), one of the components of the peripheral nervous system. G. Cellularizing embryo expressing p120-GFP fixed and stained with antibodies to the centrosomal component centrosomin (left panel, red in composite). p120-GFP was visualized by GFP fluorescence (Middle panel, green in composite). The two proteins clearly co-localize to centrosomes.

Fig. 3. The p120 region. We have mapped p120 to region 41C of chromosome arm 2R, at the junction between the euchromatin and heterochromatin. At the top is the genetic map of the region, showing complementation groups we or others have defined in the region. The heterochromatic regions and bands of region 41 are indicated. At the bottom is a representation of a portion of the physical map, with coordinates in kilobases, and a subset of the open reading frames in the region indicated. The oval indicates the centromere, which is outside of the sequenced region. *p120* represents the most proximal sequenced gene on 2R, and thus is 68 kilobases from the end of the sequenced region. There are gaps in the sequence of this region, rendering the true distances somewhat uncertain—genetics suggests that one of these gaps contains the gene *NippedB*. Between the genetic and physical maps are a set of chromosomal Deficiencies in the *Dp120* region of chromosome arm 2R. Complementation among Deficiencies and between Deficiencies and alleles from our screen has been carried out, resulting in the positioning of the Deficiencies as shown. For a subset of the Deficiencies (see text), we have also carried out PCR experiments to determine which of the open reading frames indicated on the physical map they delete.

Fig. 1 Localization of p120 during embryogenesis

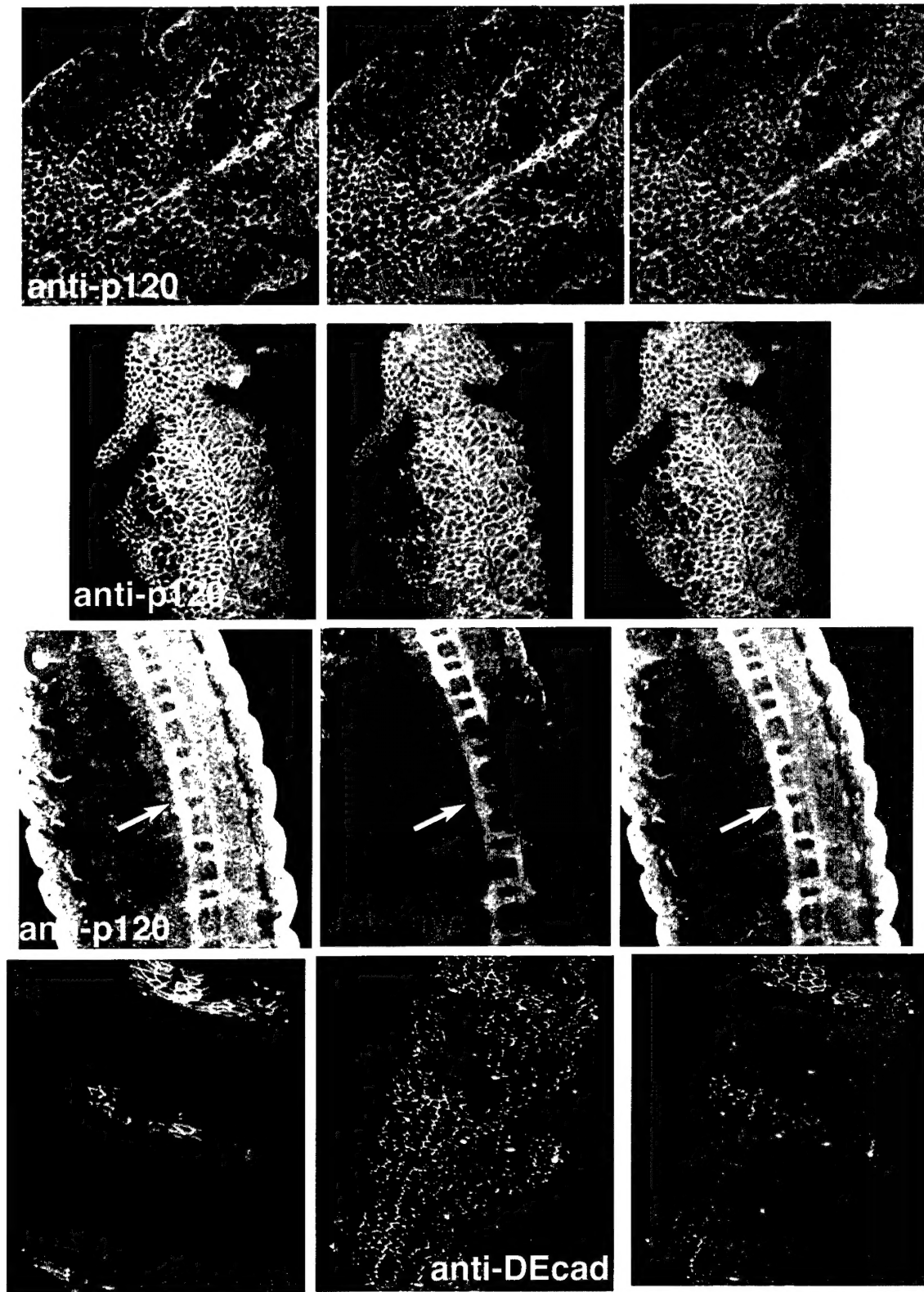
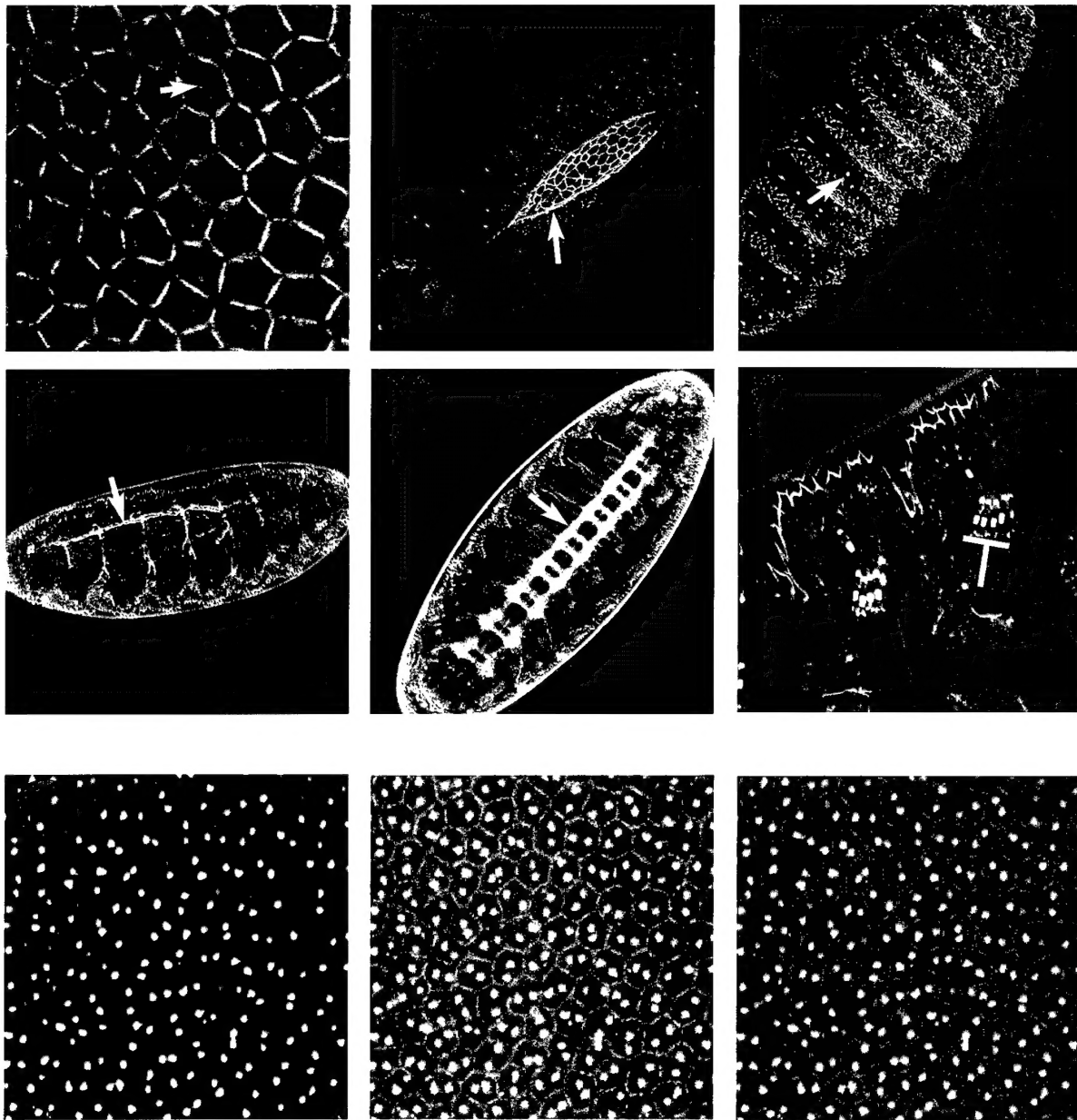


Figure 2. p120-GFP localization during embryogenesis



centrosomin

Fig. 3 The p120 region

